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Abbreviations:

A, leaf CO_2 assimilation; ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase; C_i, intercellular CO_2 concentration; DAB, 3,3´diaminobenzidine; ETR, apparent electron transport rate; g_S, stomatal conductance; GSH, glutathione; GSNO, *S*-nitrosoglutathione; MDA, malondialdehyde; NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PPFD, photosynthetic photon flux density; PSII, photosystem II; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SOD, superoxide dismutase; WD, water deficit.

Abstract

Water deficit is a major environmental constraint on crop productivity and performance and nitric oxide (NO) is an important signaling molecule associated with many biochemical and physiological processes in plants under stressful conditions. This study aims to test the hypothesis that leaf spraying of *S*-nitrosoglutathione (GSNO), a NO donor, improves the antioxidant defense in both roots and leaves of sugarcane plants under water deficit, with positive consequences for photosynthesis. In addition, the role of key photosynthetic enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate carboxylase (PEPC) in maintaining CO_2 assimilation of GSNO-sprayed plants under water deficit were evaluated. Sugarcane plants were sprayed with water or GSNO 100 μM and subjected to water deficit, by adding polyethylene glycol (PEG-8000) to the nutrient solution. Sugarcane plants supplied with GSNO presented increases in the activity of antioxidant enzymes such as SOD in leaves and CAT in roots, indicating higher antioxidant capacity under water deficit. Such adjustments induced by GSNO were sufficient to prevent oxidative damage in both organs and were associated with better leaf water status. As consequences, GSNO spraying alleviated the negative impact of water deficit on stomatal conductance and photosynthetic rates, with plants also showing increases in Rubisco activity under water deficit.

Keywords: Antioxidant enzymes; Drought; Photosynthesis; Nitric oxide; *Saccharum*.

Introduction

Low water availability is the main abiotic stress affecting sugarcane metabolism and reducing crop yield and biomass production (Ribeiro et al. 2013, Sales et al. 2013). Sugarcane plants facing water deficit present reductions in CO_2 assimilation, transpiration, stomatal opening, and decreases in tillering and culm length (Machado et al. 2009, Silva et al. 2012). While stomatal closure reduces CO_2 diffusion into mesophyll and then limit sugarcane photosynthesis under mild water deficit, metabolic limitation of photosynthesis occurs under severe water deficit through decreases in the activity of key photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate carboxylase (PEPC) (Ghannoum 2009, Lopes et al. 2011). These responses may be associated with impairment of ATP and NADPH synthesis, reducing regeneration of both ribulose-1,5-bisphosphate (RuBP) and phosphoenolpyruvate (PEP) in C_4 plants (Lawlor 2002). In fact, photochemical impairment leading to low ATP and NADPH synthesis is found under drought and it is a result of reduced electron transport rate between PSII and PSI and damage to thylakoid membranes (Lawlor and Cornic 2002).

As biochemical reactions are more sensitive to drought than photochemical ones (Sales et al. 2015) and light energy continues to be absorbed by leaves, there is a change in the redox state of the cells. Thus, the over-reduction of the electron transport chain in chloroplasts and consequent increases in electron flow to O_2 will lead to the generation of reactive oxygen species (ROS). The increase in superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) concentrations in plant cells is one of the first symptoms of exposure to drought, often accompanied by lipid peroxidation in cell membranes (Yamamoto et al. 2002, Tian and Lei 2006). To prevent or reduce cell damage due to ROS, plants have an antioxidant system based

77 on enzymatic and non-enzymatic reactions. While the non-enzymatic reactions are mediated
78 by ascorbate, glutathione, flavonoids, carotenoids and tocopherols, the enzymatic mechanism
79 consists of several enzymes located in different cellular compartments such as superoxide
80 dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). During cell
81 detoxification, $O_2^{\cdot -}$ produced in the mitochondria, chloroplasts and peroxisomes is dismuted
82 to H_2O_2 by SOD, which is rapidly eliminated by the CAT and APX, producing H_2O and O_2
83 (Foyer and Noctor 2005, Wu et al. 2012, Huseynova 2012).

84 Some studies have reported nitric oxide (NO) action in plants under drought (Santisree
85 et al. 2015, Farnese et al. 2015), with this molecule cooperating in ABA-induced stomatal
86 closure (Neill et al. 2003) and also increasing drought tolerance in *Vicia faba* (Garcia-Mata
87 and Lamattina 2001). In fact, NO is an important plant messenger mediating various
88 physiological and biochemical processes, which can directly alter protein structure and
89 activity (Cai et al. 2015). NO has a multifunctional role and its effects on plants can be
90 beneficial or not, depending on its concentration and type of donor when supplied. The *S*-
91 nitrosoglutathione (GSNO) is an important donor of NO, which is a *S*-nitrosated derivative
92 of the most abundant cellular thiol, glutathione (GSH). GSNO has been considered an
93 internal source of NO and as an essential component of NO-dependent signal transduction
94 pathway (Broniowska et al. 2013). Recently, we found increases in root growth and
95 maintenance of leaf water status in sugarcane plants under water deficit and supplied with
96 NO. GSNO-sprayed plants exhibited increases in photosynthesis under water deficit, which
97 was a consequence of high stomatal conductance and increased apparent carboxylation
98 efficiency (Silveira et al. 2016).

99 While high *S*-nitrosothiols content in GSNO-sprayed sugarcane plants suggests a long-
100 term role of NO-mediated responses to water deficit (Silveira et al. 2016), it is unclear

whether improved photosynthesis is related to the activity of key photosynthetic enzymes Rubisco and PEPC. Few studies have addressed the effects of NO on Rubisco and PEPC activities, which are likely dependent on plant species and NO donor. For instance, Kovacs (2013) showed that NO can *S*-nitrosated Rubisco in a dose-dependent manner, reducing its activity.

Alternatively, improved performance of NO-supplied plants under drought may be due to reduced oxidative damage. NO can prevent oxidative damage by activation of antioxidant mechanisms and maintenance of ROS homeostasis in two turfgrass species (Hatamzadeh et al. 2014), increasing ascorbate levels and activity of antioxidant enzymes (Zhang et al. 2008). Furthermore, $O_2^{\cdot -}$ can react with NO to form peroxynitrite (ONOO-) and therefore control ROS accumulation (Radi et al. 2002). The protective effect of exogenous application of NO donor molecules has been attributed to the elimination of $O_2^{\cdot -}$ and to increases in the activities of SOD, CAT and APX (Zhao et al. 2008). However, the consequences of such metabolic changes on photosynthetic metabolism remain unexplored. The activity of NADPH oxidase is also an important source of ROS under abiotic stress (Marino et al. 2012), with its activity reduced through *S*-nitrosylation mediated by NO (Yun et al. 2011).

While there is ample evidence of the involvement of NO in plant responses to drought, our knowledge about the underlying physiological processes leading to improved performance is still limited. In such context and using GSNO as a donor of NO, we hypothesized that leaf GSNO spraying improves the antioxidant defense of sugarcane plants under water deficit, with positive consequences for photosynthesis during and after such stressful condition. In addition, the role of key photosynthetic enzymes Rubisco and PEPC in maintaining CO_2 assimilation in GSNO-sprayed plants under water deficit was evaluated.

Material and methods

Plant material and experimental conditions

Sugarcane plants (*Saccharum* spp.) cv. IACSP94-2094 developed by ProCana Program (Agronomic Institute, IAC, Brazil) were propagated by placing mini-stalks from adult plants in trays containing commercial substrate composed of *Sphagnum*, rice straw and perlite in 7:2:1 ratio (Carolina Soil of Brazil, Vera Cruz RS, Brazil). Three-week-old plants with two to three leaves were transferred to modified Sarruge (1975) nutrient solution (15 mmol L^{-1} N (7% as NH_4^+); 4.8 mmol L^{-1} K; 5.0 mmol L^{-1} Ca; 2.0 mmol L^{-1} Mg; 1.0 mmol L^{-1} P; 1.2 mmol L^{-1} S; 28.0 µmol L^{-1} B; 54.0 µmol L^{-1} Fe; 5.5 µmol L^{-1} Mn; 2.1 µmol L^{-1} Zn; 1.1 µmol L^{-1} Cu and 0.01 µmol L^{-1} Mo) and maintained hydroponically in a growth chamber (PGR15, Conviron, Winnipeg MB, Canada) at 30/20 ºC (day/night), 80% relative humidity, 12 h photoperiod (7:00 to 19:00 h) and PPFD of 800 µmol m^{-2} s^{-1}. This PPFD was the maximum intensity at plant level, inside growth chamber. Plants were grown under the above conditions for 30 days prior to water deficit and GSNO spraying.

Sugarcane plants were subjected to water deficit (WD) by adding polyethylene glycol (Carbowax™ PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a gradual decrease in its osmotic potential as follows: -0.25 MPa on the first day; -0.5 MPa on the second day; -0.6 MPa from the third to 5th day; and -0.75 MPa from the 6th to 12th day of treatment. From this point, the osmotic potential of the nutrient solution was monitored daily and corrected when necessary. After six days under water deficit (-0.75 MPa), plants were transferred to the original nutrient solution (-0.15 MPa) for rehydration for three days.

149 Sugarcane leaves were sprayed twice a day with freshly prepared GSNO solutions at
150 100 μM. This concentration was based on a previous study in which significant increase of
151 photosynthesis was found in sugarcane plants under water deficit (Silveira et al. 2016). Leaf
152 sprays (20 mL per plant) were performed at the following days: when the osmotic potential
153 of the nutrient solution reached -0.25, -0.5 and -0.6 MPa and the last spray was done three
154 days after reaching -0.75 MPa, i.e., sprays were done on the first, second, third and 9th day
155 (Suppl. Fig. S1). GSNO spraying was done outside the growth chamber to avoid undesirable
156 interference with other treatments. Plants were then subjected to the following treatments:
157 control, in which well-hydrated plants were sprayed with water; water deficit imposed by the
158 osmotic potential of -0.75 MPa (WD) + water spray; and WD + GSNO spray (WDG). The
159 physiological evaluations as leaf gas exchange, leaf relative water content (RWC) and
160 chlorophyll content were performed at 0, 4, 12 and 15 days, which correspond to the osmotic
161 potential of -0.15 MPa (well-hydrated), -0.6 MPa, -0.75 MPa (maximum stress, 12th day) and
162 -0.15 MPa (recovery), respectively. For the enzymatic analyses, leaf and root sampling was
163 done at the maximum water deficit (12th day) and also at the recovery period (15th day).
164 Samples were collected, immediately immersed in liquid nitrogen and then stored at -80◦C.
165
166 **Synthesis of *S*-nitrosoglutathione (GSNO)**
167
168 GSNO was synthesized and characterized as previously described (Silveira et al.
169 2016). Briefly, the reduced glutathione (GSH) was reacted with equimolar sodium nitrite in
170 acidified aqueous solution, acetone was added, and the solution was washed with cold
171 water, filtered and the obtained GSNO was freeze-dried for 24 h.
172

Leaf gas exchange and photochemical activity

Gas exchange of the first fully expanded leaf with visible ligule was measured daily using an infrared gas analyzer (Li-6400, Licor, Lincoln NE, USA) attached to a modulated fluorometer (6400-40 LCF, Licor, Lincoln NE, USA). Leaf CO_2 assimilation (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured under PPFD of 2000 μmol m^{-2} s^{-1} and air CO_2 concentration of 400 μmol mol^{-1}. The measurements were performed between 11:00 and 13:00 h, as done by Silveira et al. (2016). The vapor pressure difference between leaf and air (VPDL) was 2.2±0.3 kPa and leaf temperature was 29±1 ºC during the evaluations.

Chlorophyll fluorescence was evaluated simultaneously to the leaf gas exchange and the apparent electron transport rate (ETR) was estimated as ETR= ϕ_{PSII} ×PPFD×0.85×0.4, in which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light absorption and 0.4 is the fraction of light energy partitioned to PSII (Edwards and Baker 1993, Baker 2008). The non-photochemical quenching was calculated as NPQ=(F_M − F_M')/F_M'. The relative energy excess at PSII level was calculated as EXC = [(F_V/F_M) − ϕ_{PSII}]/(F_V/F_M), in which F_V/F_M is the potential quantum efficiency of photosystem II (Bilger et al. 1995). A chlorophyllmeter (CFL 1030, Falker, Porto Alegre RS, Brazil) was used to evaluate chlorophyll *a* and *b* and the relative content of chlorophyll (Chl) was calculated as chlorophyll *a* + *b*.

Measurements were taken at the beginning of the experiment (before reducing the osmotic potential of nutrient solution), when the osmotic potential of nutrient solution reached -0.75 MPa, at the maximum water deficit, and three days after returning plants to the control condition (recovery period).

197

198 **Relative water content**

199

200 The relative water content (RWC) was calculated in leaf discs according to Jamaux et
201 al. (1997): RWC=100*[(FW-DW)/(TW-DW)], in which FW, TW and DW are the fresh,
202 turgid and dry weights, respectively.

203

204 **Activity of photosynthetic enzymes**

205

206 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39)

207

208 Approximately 100 mg of leaves were macerated and homogenized with 0.5 mL of 100
209 mM bicine-NaOH buffer (pH 7.8), 1 mM ethylenediaminetetraacetic (EDTA), 5 mM $MgCl_2$,
210 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μM
211 leupeptin. The resulting solution was centrifuged at 14000 *g* for 5 min at 4 °C. The initial
212 activity of Rubisco was measured using as medium 100 mM bicine-NaOH (pH 8.0)
213 containing 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM
214 NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric
215 phosphokinase and 80 nkat creatine phosphokinase, incubated at 25 °C. The oxidation of
216 NADH was initiated by adding 0.5 mM ribulose-1,5-bisphosphate (RuBP). A similar aliquot
217 of clarified leaf extract was incubated with the reaction medium for 10 min at 25 °C and total
218 Rubisco activity was measured after adding RuBP. The reduction of absorbance at 340 nm
219 was monitored for 3 min (Sage et al. 1988, Reid et al. 1997). The activation state of Rubisco

was calculated as the ratio between the initial and total activities and the main panel shows the initial activity of the Rubisco.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31)

Approximately 100 mg of leaves were macerated and homogenized with 0.5 mL of 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA and 1 mM PMSF and centrifuged at 14000 *g* for 25 min at 4 °C and the supernatant collected. The reaction medium for PEPC activity contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 10 mM $NaHCO_3$, 33 nkat malic dehydrogenase and 0.3 mM NADH. The reaction was initiated by adding 4 mM phosphoenolpyruvate and incubated at 30 °C. The oxidation of NADH was monitored for 1 min (Degl'innocenti et al. 2002).

Reactive oxygen species

The quantification of hydrogen peroxide (H_2O_2) in plant material was performed following the method of Alexieva et al. (2001). Homogenates were obtained from 0.1 g of fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was centrifuged at 10000 *g* and 4 °C for 15 min. The reaction medium consisted of 1.2 mL of 1 mM KI, 0.1 M potassium phosphate buffer (pH 7.5) and crude extract. The microtubes were incubated on ice under dark for 1 h. After this period, the absorbance was read at 390 nm. A standard curve was obtained with H_2O_2 and the results were expressed as μmol H_2O_2 g^{-1} FW.

To determine the concentration of the superoxide anion ($O_2^{\cdot -}$), 50 mg of samples were incubated in an extraction medium consisting of 100 µM EDTA, 20 µM NADH, and 20 mM sodium phosphate buffer, pH 7.8 (Kuo and Kao 2003). The reaction was initiated by adding 100 µL of 25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28 °C under stirring for 5 min and the absorbance was read at 480 nm for 5 min. Superoxide anion production was assessed by the accumulation of adrenochrome using a molar absorption coefficient of 4.0×10^3 M^{-1} (Gay and Gebicki 2000).

Lipid peroxidation

The concentration of malondialdehyde (MDA) was measured and used as a proxy of lipid peroxidation. One hundred fifty mg of plant tissue were macerated in 2 mL of 0.1% TCA (w/v) and centrifuged at 10000 *g* for 15 min. The supernatant was added to 1.0 mL of 0.5 % thiobarbituric acid (w/v) in 20% TCA (w/v), and the mixture was incubated at 95 °C. Two hours after the reaction, the absorbance was measured at 532 and 600 nm. The absorbance at 600 nm was subtracted from the absorbance at 532 nm and the MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Protein extraction and antioxidant activity

Plant extracts were obtained from the macerate of 0.1 g of fresh tissue (roots or leaves) with liquid nitrogen, 1% PVPP and 2 mL of extraction medium containing 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM EDTA and 1 mM PMSF. After centrifugation of the

267 homogenates at 15000 *g* for 15 min and 4 ºC, supernatants (crude extracts) were collected
268 and preserved on ice. The protein levels of the enzymatic extracts were determined by the
269 Bradford method (Bradford 1976) using bovine serum albumin (BSA) as the standard.

270 Catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure described
271 in Havir and McHale (1987). The reaction medium consisted of 3 mL of 100 mM potassium
272 phosphate buffer (pH 6.8), deionized water, 125 mM H_2O_2 and crude extract. The reaction
273 was carried out in a water bath at 25 ºC for 2 min and activity assessed by the decrease in
274 absorbance at 240 nm. The CAT activity was calculated using the molar extinction
275 coefficient of 36 M^{-1} cm^{-1} and expressed as nmol min^{-1} mg^{-1} of protein.

276 Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as described by
277 Nakano and Asada (1981). The reaction medium was composed by 3 mL of 100 mM
278 potassium phosphate buffer (pH 6.0), deionized water, 10 mM ascorbic acid, 10 mM H_2O_2
279 and crude extract. The reaction was carried out in a water bath at 25 ºC for 2 min and activity
280 monitored by the decrease in absorbance at 290 nm, using the molar extinction coefficient of
281 2.8 M^{-1} cm^{-1} to measure the APX activity, which was expressed as μmol min^{-1} mg^{-1} of
282 protein.

283 Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to
284 Giannopolitis and Ries (1977). The reaction medium consisted of 3 mL of 100 mM sodium
285 phosphate buffer (pH 7.8), 50 mM methionine, 5 mM EDTA, deionized water, crude extract,
286 100 μM riboflavin and 1 mM nitro blue tetrazolium chloride (NBT). A group of tubes was
287 exposed to light (fluorescent lamp of 30 W) for 8 min, and another group remained in
288 darkness. The absorbance was measured at 560 nm and one unit of SOD is the amount of
289 enzyme required to inhibit the NBT photoreduction in 50%, being expressed as U min^{-1} mg^{-1}
290 of protein.

291

292 **Superoxide dismutase zymogram**

293

294 In order to check the cellular compartment in which the first line of enzymatic defense
295 against oxidative damage was active, the identification of SOD isoforms was performed by
296 native-PAGE electrophoresis in polyacrylamide gel (12.5%) at 4 ºC under constant current
297 of 40 mA in Tris-HCl (pH 8.3) and glycine buffer. The protein extract was obtained after
298 maceration of a composite leaf sample (0.4 g, composed by ~0.1 g per replicate) (Ferreira et
299 al. 2002). SOD isoforms were identified through specific inhibition by H_2O_2 and KCN, with
300 isoforms being classified as Mn-SOD (resistant to both inhibitors), Fe-SOD (resistant only
301 to KCN) and Cu/Zn-SOD (inhibited by both inhibitors) (Azevedo et al. 1998).

302

303 **Histochemical analysis**

304

305 Samples of three leaves per plant (1 cm^2) in three plants per treatment were cut and
306 immediately immersed in a solution of 1 mg mL^{-1} of DAB-HCl, adjusted to pH 5.6 with
307 NaOH and incubated in dark for 8 h. Samples were taken at the maximum water deficit and
308 they were cleared in 96% ethanol (Faoro and Iriti 2005) before transversal hand cuts. Samples
309 were examined with a light microscope Olympus BX41 (Tokyo, Japan) fitted with a digital
310 camera (Media Cybernetics PL-A624, Bethesda MD, USA). H_2O_2 was visualized as a
311 reddish-brown coloration. As a negative control, DAB solution was supplemented with 10
312 mM ascorbic acid (Faoro et al. 2001).

313

314 **Data analysis**

315

316 The experimental design was completely randomized and data were subjected to the
317 ANOVA procedure. The Scott-Knott test was used to compare treatments when significance
318 was found ($P<0.05$). The results presented are the mean ± SD and the number of replicates
319 is stated in each figure caption.

320

321 **Results**

322

323 **GSNO improves leaf water status and chlorophyll content under water deficit**

324

325 As compared to well-hydrated plants, water deficit caused significant reduction in
326 relative water content (RWC) of sugarcane plants at the maximum water deficit. Such
327 reduction in RWC was alleviated when plants were sprayed with GSNO (Fig. 1A). Low water
328 availability also reduced the chlorophyll content of sugarcane plants at the maximum water
329 deficit and recovery period. Again, GSNO spraying alleviated the decreases in chlorophyll
330 content caused by water deficit (Fig. 1B).

331

332 **GSNO improves leaf gas exchange, photochemistry and increases Rubisco activity**
333 **under water deficit**

334

335 The water deficit induced a large reduction in leaf CO_2 assimilation of sugarcane plants
336 from the 4th day of treatment, with GSNO treatment improving leaf CO_2 assimilation during
337 the water deficit (Fig. 2A). The stomatal conductance was affected by water deficit and
338 GSNO supplying in a similar way as compared to leaf CO_2 assimilation (Fig. 2B). There was

339 a significant increase in intercellular CO_2 concentration (C_i) at the maximum water deficit
340 when plants were not supplied with GSNO and subjected to water deficit (Fig. 2C). The
341 apparent electron transport rate (ETR) was reduced due to water deficit. However, such
342 negative effect of water deficit was nulled by GSNO only at the beginning of water deficit
343 treatment (Fig. 2D). The consumption of electrons per CO_2 assimilated (ETR/*A*) was
344 increased in plants under water deficit conditions. However, plants sprayed with GSNO
345 presented lower ETR/*A* than plants under low water availability and without GSNO
346 supplying, mainly at the maximum water deficit (Fig. 2E). Leaf GSNO spraying caused the
347 lowest non-photochemical quenching at the maximum water deficit (Fig. 2F). While water
348 deficit caused increases in excessive light energy at PSII (EXC), GSNO spraying led to
349 reductions in EXC and then control plants and those treated with GSNO presented similar
350 values during the first days of water deficit (data not shown).
351 Rubisco activity was decreased by 65% in plants under water deficit and increased
352 above 82% from controls by GSNO supplying (Fig. 3A). On average, GSNO-supplied plants
353 presented Rubisco activity almost two-fold higher than control ones at the maximum water
354 deficit (Fig. 3A). On the other hand, the Rubisco activation state (Fig. 3A, insert) and PEPC
355 activity were not affected by water deficit or GSNO supplying (Fig. 3B).
356
357 **GSNO and oxidative damage**
358
359 Even without a statistical difference in leaf H_2O_2 concentration among treatments (data
360 not shown), the histochemical analysis revealed a local H_2O_2 accumulation in plants under
361 water deficit and without GSNO supplying (Fig. 4B). H_2O_2 accumulation occurred in the
362 mesophyll cells near to the stomatal guard cells, as indicated by a brownish precipitate

staining cell walls and chloroplasts. Such H_2O_2 accumulation was not noticed in WDG and control plants (Fig. 4A,C). At the maximum water deficit and recovery period, the highest $O_2^{\cdot-}$ concentrations in both leaves and roots were found in plants subjected to water deficit. Interestingly, plants supplied with GSNO and control ones presented similar leaf and root $O_2^{\cdot-}$ concentrations (Fig. 5A,B). Leaf lipid peroxidation was not changed by treatments. However, there was an increase in root MDA content of plants under water deficit without GSNO supplying at the recovery period (Fig. 5C,D).

GSNO stimulates the antioxidant system under water deficit

Leaf CAT activity decreased due to water deficit, regardless whether GSNO was supplied or with evaluation time (Fig. 6A). In plants sprayed with GSNO, root CAT activity increased by 44% at the maximum water deficit and by 49% at the recovery period, as compared to control ones (Fig. 6B). At the maximum water deficit, leaf APX activity was increased only in plants subjected to water deficit and without GSNO treatment (Fig. 6C). In roots, APX activity decreased due to water deficit and GSNO spraying did not modify this response at the maximum water deficit and recovery period (Fig. 6D). Compared to the control condition, leaf SOD activity was significantly increased by GSNO spraying at the maximum water deficit (Fig. 6E). Root SOD activity was increased by water deficit, regardless GSNO spraying and evaluation time (Fig. 6F).

The SOD zymogram revealed details about the activities of SOD isoforms, which were numbered arbitrarily based on the order of appearance on the gels. When considering leaf SOD isoforms, five isoforms were identified in control treatment and seven in water deficit, regardless of GSNO spraying (Fig. 7). There were four and seven root SOD isoforms in

387 control and water deficit conditions, respectively. In leaves, Cu/Zn-SOD isoforms *iii* and *iv*
388 were detected only under water deficit, regardless of GSNO spraying. However, GSNO
389 increased the activity of all SOD isoforms when compared to the other treatments. In roots,
390 Mn-SOD isoform *iii* and Cu/Zn-SOD isoforms *v* and *vi* were detected only in plants under
391 water deficit, regardless of GSNO supplying (Fig. 7).
392
393 **Discussion**
394
395 **How does GSNO improve photosynthesis under water deficit?**
396
397 Our findings clearly show that leaf GSNO spraying improves sugarcane tolerance to
398 water deficit by improving photosynthetic rates. Such improvement in leaf CO_2 assimilation
399 was associated, in part, with higher RWC and higher stomatal conductance under water
400 deficit (Figs. 1A, 2B). For instance, GSNO treatment increased by seven times the stomatal
401 conductance at the maximum water deficit as compared to plants sprayed with water (Fig.
402 2B). It could be argued that improvements in leaf water status were concomitant with the
403 stomatal conductance in GSNO-sprayed plants under water deficit, which was lower than in
404 control plants (Fig. 2B). As consequence, GSNO-sprayed plants would minimize water vapor
405 loss through transpiration (Suppl. Fig. S2) and maximize CO_2 availability in intercellular
406 spaces. Accordingly, control plants and those sprayed with GSNO presented similar C_i values
407 throughout the experimental period (Fig. 2C).
408 Besides the diffusive limitation of photosynthesis imposed by stomatal closure under
409 water deficit, plants may face biochemical limitation under severe drought. Then, GSNO
410 would also improve CO_2 uptake under water deficit through changes in the activity of key

411 photosynthetic enzymes. In fact, plants supplied with GSNO exhibited an increase in Rubisco
412 activity under water deficit (Fig. 3A). Beligni and Lamattina (2002) found that the NO
413 delayed Rubisco mRNA loss caused by treatment with the herbicide Diquat in potato leaves.
414 On the other hand, NO can also *S*-nitrosylate Rubisco causing reduced Rubisco activity in a
415 dose-dependent manner (Kovacs et al. 2013, Santisree et al. 2015). As possible explanation
416 for contradictory effects of NO on Rubisco, we would consider the NO donor and its
417 concentration as well as plant sensitivity, which likely vary among plant species. By spraying
418 GSNO 100 μM, our data clearly show improvements in sugarcane photosynthesis through
419 different techniques and approaches, i.e., measuring leaf gas exchange and using biochemical
420 assays. For a complete picture of how GSNO improves sugarcane photosynthesis, we should
421 understand the regulation of photosynthetic enzymes through nitration and/or *S*-nitrosylation
422 and its impact on plant metabolism under water deficit.

423 GSNO also alleviated chlorophyll degradation (Fig. 1B), which is in agreement with
424 previous studies showing that exogenous application of NO may attenuate chlorophyll
425 degradation under stressful conditions (Eum et al. 2009, Wang et al. 2015). Accordingly,
426 plants sprayed with GSNO presented higher photochemical activity under water deficit as
427 compared to those sprayed with only water and also subjected to water deficit. Besides
428 showing ETR values similar to control plants during the beginning of water deficit (Fig. 2D),
429 plants sprayed with GSNO presented less activity of alternative electrons sinks under water
430 deficit (Fig. 2E). Low non-photochemical quenching of chlorophyll fluorescence also
431 suggests less excessive light energy at the PSII level (Fig. 2F).

432 Taken together, we may argue that GSNO spraying benefited plants through
433 improvements in leaf water status, which may be a consequence of better water balance

caused by increases in water supply by roots and/or reduced water loss by stomatal conductance while maintaining CO_2 availability for photosynthesis. Such higher photosynthetic rates may also be due to increases in Rubisco activity in GSNO-sprayed plants with an important regulatory role at the PSII level as the main sink of ATP and NADPH. While less energetic pressure at PSII level can be maintained by using of reducing power and ATP in CO_2 assimilatory reactions under low water availability, an active antioxidant system would also prevent oxidative damage induced by drought. Therefore, GSNO effects on antioxidant metabolism were further investigated in both roots and leaves of sugarcane under water deficit.

GSNO improves antioxidant defense in both roots and leaves under water deficit

Water deficit induced leaf $O_2^{\cdot-}$ accumulation (Fig. 5A) without a concomitant increase in SOD activity (Fig. 6E) in plants without GSNO spraying. Presence of H_2O_2 in leaves of plants under water deficit (Fig. 4B) was also found even with increases in APX activity (Fig. 6C). As CAT activity was reduced and leaves presented H_2O_2 under water deficit, one would argue that the main H_2O_2 detoxification pathway in sugarcane is through CAT activity. In roots, accumulation of $O_2^{\cdot-}$ was also found under water deficit, with increases in SOD activity and decreases in APX activity (Figs. 5A, 6D,F). Interestingly, GSNO-sprayed plants did not present any increase in $O_2^{\cdot-}$ and presence of H_2O_2 in leaves (Figs. 4, 5A,B) and GSNO stimulated leaf SOD activity and root CAT activity under water deficit (Figs. 4C, 6B,E).

The increase in total SOD activity (Fig. 6E) in leaves sprayed with GSNO was associated with increases in the activity of isoforms (Fig. 7) found in cellular compartments such as mitochondria (Mn-SOD), peroxisomes (Mn-SOD and Cu/Zn-SOD), chloroplasts

458 (Fe-SOD and Cu/Zn-SOD), cytosol (Cu/Zn-SOD) and apoplast (Cu/Zn-SOD) (Alscher et al.
459 2002). As a product of SOD activity, accumulation of H_2O_2 in leaves would be expected.
460 However, evidence of H_2O_2 production was not found in GSNO-sprayed plants under water
461 deficit. It is known that the detoxification of H_2O_2 can also be achieved by non-enzymatic
462 antioxidants such as non-protein thiol, ascorbate, GSH or proline. The protective effect of
463 GSH could not be discarded in plants sprayed with GSNO and subjected to water deficit.
464 GSNO is decomposed by GSNO reductase to oxidized glutathione (GSSG), which is the
465 substrate of the glutathione reductase (GR) that regenerates GSH (Corpas et al. 2013). In fact,
466 GSH spraying improved photochemical activity in sugarcane leaf discs subjected to natural
467 drying (Silveira et al. 2016).

468 Although we have not found evidence of membrane damage under water deficit in both
469 leaves and roots (Figs. 5C,D), effects of oxidative stress on protein and DNA integrity and
470 function cannot be ruled out (Oliveira et al. 2010). Leaf and root $O_2^{\cdot-}$ concentrations remained
471 high even after the recovery period in plants previously exposed to water deficit, with
472 increased lipid peroxidation in roots (Figs. 5A,B,D). The production of ROS may occur by
473 the action of NADPH oxidase and peroxidases, causing an oxidative burst during rehydration
474 (Colville and Kranner 2010), which was also avoided in both leaves and roots by spraying
475 GSNO on leaves.

476 Interestingly, we have found that root enzymes were also affected due to leaf
477 applications, demonstrating the potential GSNO signaling in response to water deficit.
478 Accordingly, Silveira et al. (2016) found increases in root dry mass in plants sprayed with
479 GSNO, which improved leaf water status and the photosynthetic apparatus under water
480 deficit. A recent study demonstrated that GSNO promotes cell wall remodeling, alters
481 transcription and induces the formation of root hair in mutants of Arabidopsis thaliana. It has

also been shown that unlike auxin, GSNO affects the transcription of genes encoding ribosomal proteins and DNA-modifying enzymes and histones, such as histone acetylases, which have been shown to be the target of *S*-nitrosylation (Moro et al. 2017).

The changes observed in antioxidant metabolism indicate that leaf GSNO spraying increased the antioxidant responses of both leaves and roots, avoiding oxidative damage induced by $O_2^{\cdot-}$ and H_2O_2 under water deficit. Such responses may be related to plant water status as H_2O_2 may inhibit aquaporin activity and reduce water transport (Liu et al. 2015). In fact, higher RWC and stomatal conductance in GSNO-sprayed plants were noticed (Figs. 1A, 2B). Silva et al. (2015) have already reported that sugarcane genotypes showing an active antioxidant metabolism are able to maintain root hydraulic conductance and leaf gas exchange under water deficit. Additionally, NO by itself has been reported to play an important role in aquaporin regulation by stimulating the transcription of *Os*PIP1;1, *Os*PIP1;2, *Os*PIP1;3 and *Os*PIP2;8 isoforms during rice seed germination (Liu et al. 2007). Further studies about the potential effect of NO on plant hydraulic conductance and aquaporin expression and activity should reveal another interesting interaction among this radical, antioxidant metabolism and plant water balance.

Conclusion

Sugarcane plants supplied with GSNO presented increases in the activity of SOD in leaves and CAT in roots, indicating higher antioxidant capacity under water deficit. Such adjustments induced by GSNO were sufficient to prevent oxidative damage in both organs and were associated with better leaf water status. As consequence, GSNO spraying alleviated the negative impact of water deficit on stomatal conductance and photosynthetic rates, with

506 plants also showing increases in Rubisco activity under water deficit. Herein, we not only
507 gave one step more toward the understanding of how NO may modulate plant responses to
508 water deficit but also we provided insights about the underlying physiological processes
509 supporting improved plant performance under stressful conditions.
510
511 **Acknowledgment**
512
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520
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671 **Figure captions**

672

673 **Fig. 1.** Leaf relative water content (RWC, in A) and chlorophyll content (B) in sugarcane
674 plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water
675 (WD) or subjected to WD and sprayed with GSNO 100 μM (WDG). The shaded area
676 indicates recovery period, when plants were moved to nutrient solution used in control
677 treatment. Each symbol represents the mean value of four replications ± standard deviation.
678 Different lowercase letters indicate statistical difference between treatments (Scott-Knott
679 test, $P<0.05$).

680

681 **Fig. 2.** Leaf CO_2 assimilation (A, in A), stomatal conductance (g_S, in B), intercellular CO_2
682 concentration (C_i, in C), apparent electron transport rate (ETR, in D), ratio of electron
683 transport rate and CO_2 assimilated (ETR/A, in E), and non-photochemical quenching (NPQ,
684 in F) in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and
685 sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 μM (WDG). The
686 shaded area indicates recovery period, when plants were moved to nutrient solution used in
687 control treatment. Each symbol represents the mean value of four replications ± standard
688 deviation. Different lowercase letters indicate statistical difference between treatments
689 (Scott-Knott test, $P<0.05$).

690

691 **Fig. 3.** *In vitro* activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, in A)
692 and phosphoenolpyruvate carboxylase (PEPC, in B) in sugarcane plants maintained well-
693 hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to
694 WD and sprayed with GSNO 100 μM (WDG). Evaluations were done at the maximum water

695 deficit. In A, Rubisco activation state is shown as insert. The data represents the mean value
696 of three replications ± standard deviation. Different lowercase letters indicate statistical
697 difference between treatments (Scott-Knott test, $P<0.05$).
698
699 **Fig. 4.** Cross section of sugarcane leaves in which with H_2O_2 was stained brown by DAB. A,
700 well-hydrated (Control) samples with no H_2O_2 accumulation. B, sample subjected to water
701 deficit (WD) with intense H_2O_2 accumulation in cell walls (cw) and chloroplast (ch) of
702 mesophyll (mc) and guard cells (sgc). C, samples subjected to WD and sprayed with GSNO
703 100 µM (WDG) with no H_2O_2 accumulation. ep: epidermis, bsc: bundle-sheath cells. Bars:
704 20 µm.
705
706 **Fig. 5.** Concentration of superoxide anion ($O_2^{\cdot-}$, in A and B) and malondialdehyde (MDA in
707 C and D) in leaves (A and C) and roots (B and D) in sugarcane plants maintained well-
708 hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to
709 WD and sprayed with GSNO 100 µM (WDG). MWD indicates maximum water deficit and
710 Rec and shaded area indicate recovery period. The data represents the mean value of three
711 replications ± standard deviation. Different lowercase letters indicate statistical difference
712 between treatments (Scott-Knott test, $P<0.05$).
713
714 **Fig. 6.** Activity of catalase (CAT, in A and B), ascorbate peroxidase (APX, C and D) and
715 superoxide dismutase (SOD, in E and F) in leaves (A, C and E) and roots (in B, D and F) in
716 sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed
717 with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). MWD
718 indicates maximum water deficit and Rec and shaded area indicate recovery period. The data

719 represents the mean value of three replications ± standard deviation. Different lowercase
720 letters indicate statistical difference between treatments (Scott-Knott test, $P<0.05$).
721
722 **Fig. 7.** Superoxide dismutase isoforms by non-denaturing polyacrylamide gel electrophoresis
723 in leaves and roots in sugarcane plants maintained well-hydrated (Control), subjected to
724 water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100
725 μM (WDG). Evaluations were done at the maximum water deficit. Gels are representative of
726 three runs. Lanes were loaded with 39.5 μg of leaf protein and 17.2 μg of root protein.

727 Figure 1

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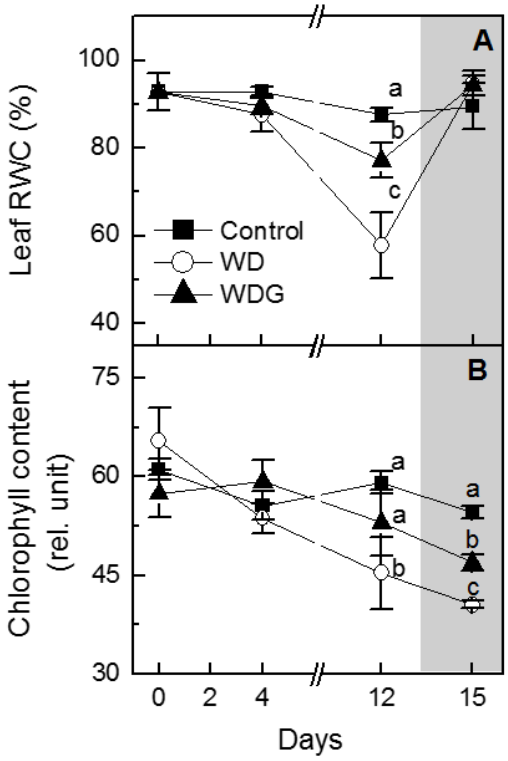

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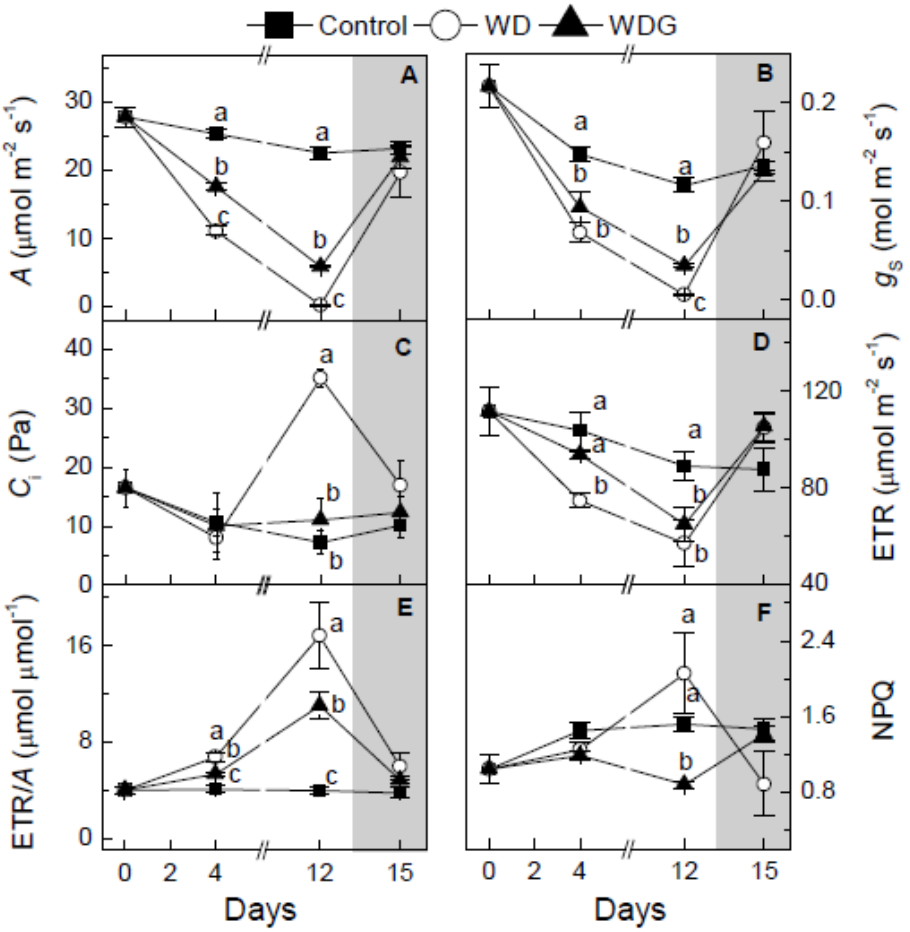
734 Figure 2



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737 Figure 3

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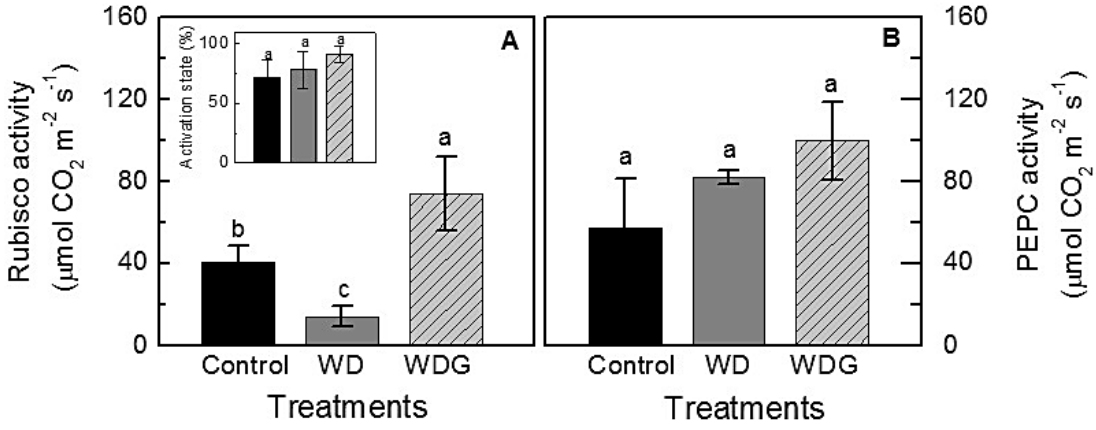

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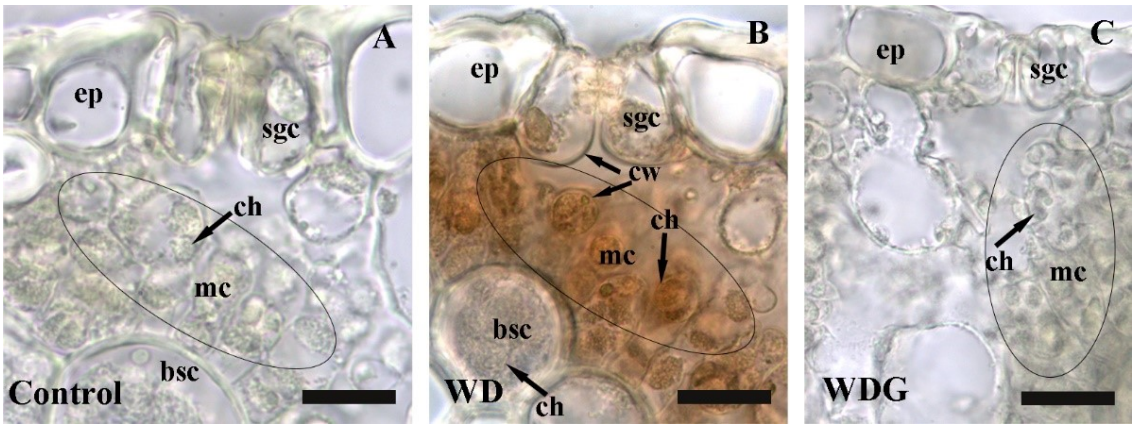
741 Figure 4



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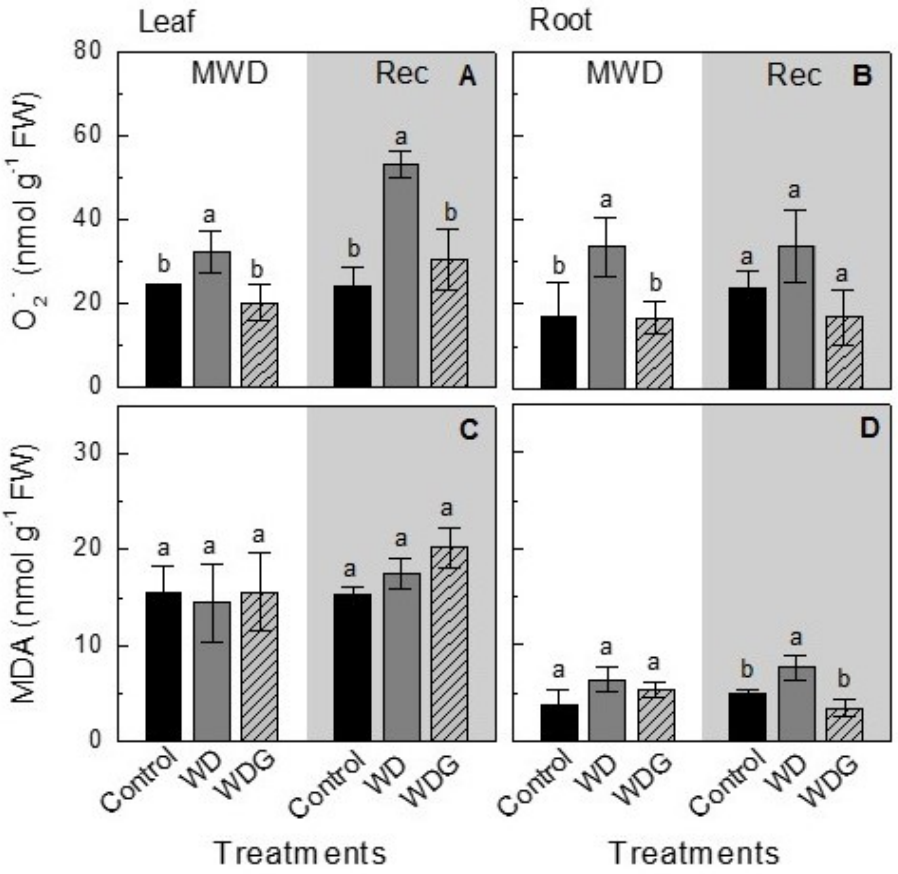
746 Figure 5



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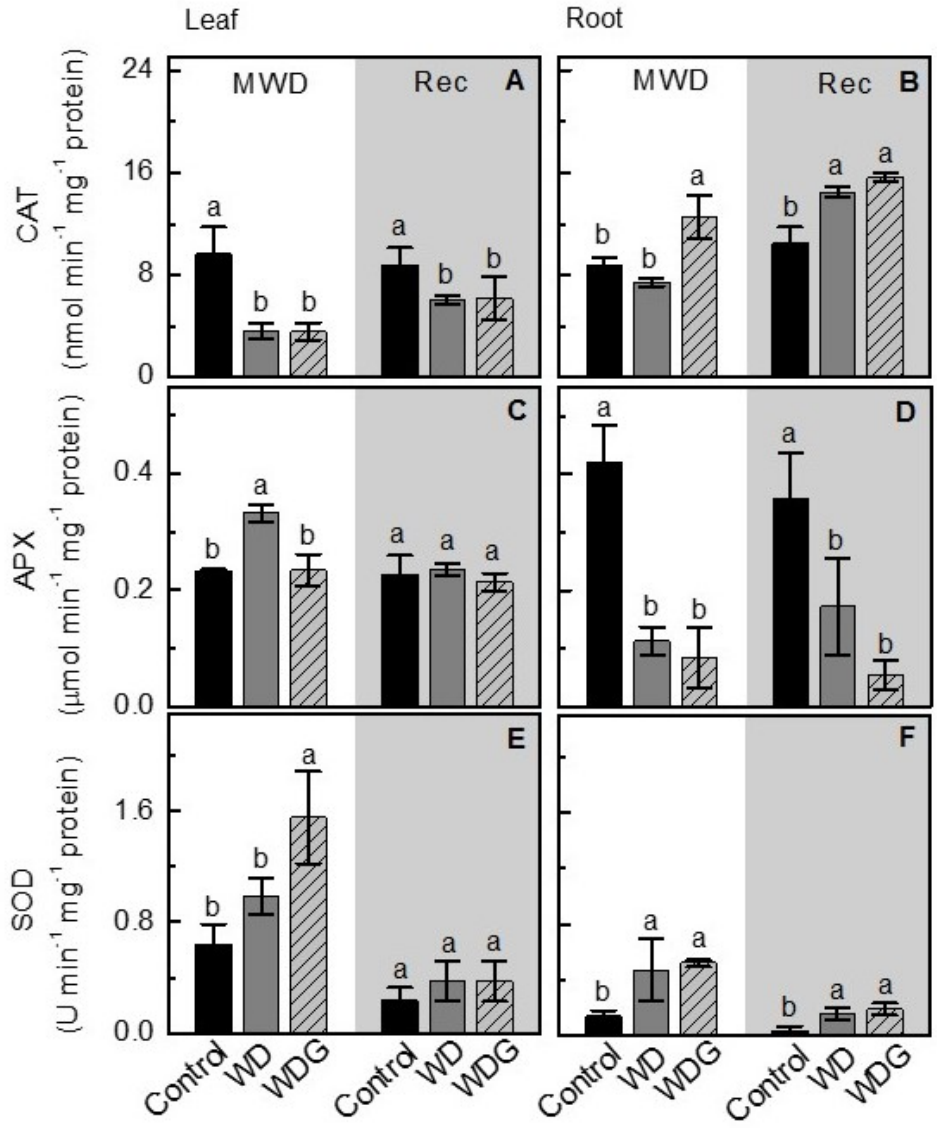
750 Figure 6



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759 Figure 7

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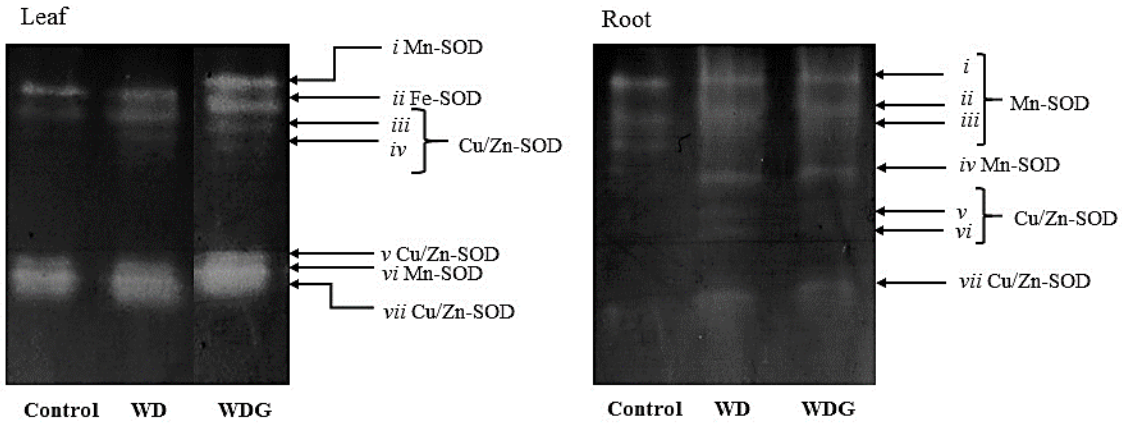

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765 **Supplementary material**

766

767 Figure S1

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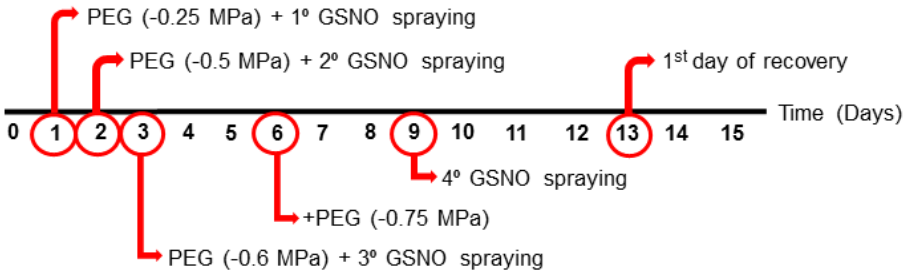

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771 **Fig S1.** Simplified scheme showing GSNO spraying and reduction of the osmotic potential
772 of nutrient solution by adding PEG-8000. PEG-8000 was added to the nutrient solution to
773 cause a gradual decrease in its osmotic potential: -0.25 MPa on the first day; -0.5 MPa on the
774 second day; -0.6 MPa from the third to 5th day; and -0.75 MPa from the 6th to 12th day of
775 treatment. Recovery means the return to the nutrient solution without PEG-8000.

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779 Figure S2

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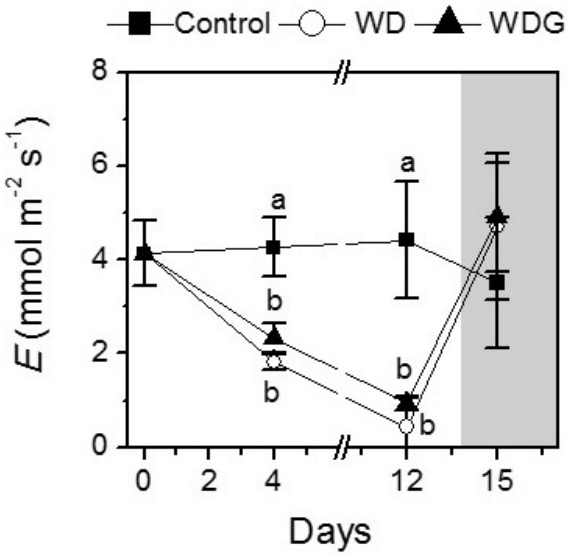

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782

783 **Fig S2.** Transpiration (*E*) in sugarcane plants maintained well-hydrated (Control), subjected
784 to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO
785 100 μM (WDG). The shaded area indicates recovery period, when plants were moved to
786 nutrient solution used in control treatment. Each symbol represents the mean value of four
787 replications ± standard deviation. Different lowercase letters indicate statistical difference
788 between treatments (Scott-Knott test, $P<0.05$).

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790